Associative Learning of an Odor to a Sugar-meal by *Anopheles gambiae* (Diptera: Culicidae)

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction* in Entomology in the undergraduate colleges of The Ohio State University

by

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December 2010

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Abstract

The abilities of many insects to learn have been well documented. However, a limited number of studies have been conducted to determine associative learning capabilities in medically important insects. To date, no studies of this sort have been carried out with the malaria vector *Anopheles gambiae*. This research study used *Culex pipiens* to investigate methods used in a previous study that looked at the learning abilities of *Cx. quinquefasciatus* to associate an odor (conditioned stimulus) with a sugar-meal (unconditioned stimulus) by individual training and testing. Those methods were then adapted and used to examine associative learning capabilities in *An. gambiae* by both individual training, and testing with a dual-port olfactometer. Vanilla and almond extracts were used for individual training and testing of *Cx. pipiens*, but were found to be unsuitable. In order to determine compounds more appropriate than extracts to train and test *An. gambiae*, two sets of experiments were carried out with chemicals found in honey to determine *An. gambiae* mosquitoes' innate responses to them. From those results, phenylacetaldehyde and geranylacetone were chosen to be used for the dual-port olfactometer trials, and linalool oxide and (Z)-β-ocimene were chosen to be used for the individual training and testing of *An. gambiae*. The results indicate the possibility that *An. gambiae* mosquitoes can associatively learn and illustrate the need to ensure that all the parameters that may affect the learning abilities and behaviors of mosquitoes are taken into account in the experimental design so that definitive conclusions about their learning abilities can be made.

Introduction

Learning is a process by which an organism benefits from experience so that its future behavior is better adapted to its environment (Rescorla, 1988). A variety of insects—fruit flies, grasshoppers, parasitoid wasps, honey bees, etc.—rely extensively on learning for all major life activities such as feeding, predator avoidance, aggression, and social interactions (Dukas, 2008).

Associative learning capabilities, which are characterized by a behavioral changes resulting from paired learning events (Mackintosh, 1974), have been shown in several insects. *Drosophila* larvae can associate odor with electric shock (Aceves-Pina and Quinn, 1979), food quality, and danger (Dukas, 1999) as well as illumination conditions with sugar, quinine, and table salt (Gerber et al, 2004). Adult *Drosophila* have been shown to be able to associate odors with electric shock (Quinn et al., 1974) and sugar water (Tempel et al., 1983), colored light with aversive states, such as shock (Folkers, 1982) or violent shaking (Quinn et al., 1974), and visual patterns with excessive heat (Wolf and Heisenberg, 1991; Liu et al., 2006). Grasshoppers have the ability to associate visual cues with nutritional qualities and use that learned information to seek out a desired nutrient (Raubenheimer and Tucker, 1997), and are able to learn to avoid foods containing harmful compounds (Lee and Bernays, 1990). Olson et al (2003) showed that a trained parasitoid wasp, *Microplitis croceipes*, can associate odors with food as well as odors, colors, shapes, and visual patterns with a host.

Honey bees have their own wide array of learned behavior. It has been demonstrated that honey bees learn to respond differentially to rewarded and unrewarded odors (Faber et al., 1999). A bee observing a waggle dance not only learns information about the direction and distance to visited flowers, but learns the associated floral odors from the forager bee (von Frisch, 1967; Seeley, 1996; Farina et al., 2005). These cues are used to find the specific flowers once the

general vicinity is reached (von Frisch, 1967; Riley et al., 2005). It has been stated by Dukas (2008) that the foraging abilities of honey bees are positively correlated with their age due to learning a variety of tasks.

Several investigators have looked at learning in the medically important mosquito. *Culex tritaeniorhynchus* and *Cx. vishnui* are more likely to feed on a host species on which they had previously fed, suggesting 'behavioral imprinting' (Mwandawiro et al., 2000). This also shows evidence of behavioral conditioning as opposed to genetic variability in the host preferences of these three species (Mwandawiro et al., 2000). It has been demonstrated that *Cx. quinquefasciatus* show a change in odor preference following exposure to odor during rearing in the aquatic stage, which suggests that some sort of larval conditioning or early adult imprinting occurred (McCall and Eaton, 2001). Tomberlin et al. (2006) reported that *Cx. quinquefasciatus* are able to learn and associate an odor with a sugar- or blood-meal and can distinguish the odor to which they were trained from other odors. Ferrari et al. (2008) demonstrated that *Cx. restuans* larvae can learn to recognize the odor of a novel predator after a single conditioning event in a threat-sensitive manner. Jhumur et al. (2006) showed that trained the *Cx. pipiens pipiens* biotype *molestus* were significantly more attracted to volatiles than naïve mosquitoes, that learning can increase the attractiveness of odors to mosquitoes, that the innate attraction to odors was positively associated with the amount of time that had passed since the last feeding, and that the amount of time the trained mosquitoes had been starved modified the strength of their response, probably due to a physiological threshold shift for nectar searching. These examples indicate that *Cx. pipiens pipiens* biotype *molestus* can retain information; ergo, they have memory (Jhumur et al., 2006).

Associative learning and memory in *Aedes aegypti* against oviposition site deterrence in repellent-laden water has been reported (Kaur et al., 2003). However, habituation or sensitization of the receptors that detect the repellent chemical could also explain the results (Alonso and Schuck-Paim, 2006). Also, no evidence to support associative learning in *Ae. aegypti* could be found by Alonso et al. (2003), but this could be due to methodological inadequacy (Alonso and Schuck-Paim, 2006).

Previous studies of *Anopheles melas* have reported possible learning by their ability to choose a pathway in a field, but it was unclear whether the mosquitoes learned by experience (acquired behavior), innate behavior, or followed their conspecifics (Giglioli, 1964; Snow, 1970). It has been proposed that learning visual cues enables *An. farauti* to find known oviposition sites without having to make long searching-flights (Charlwood et al., 1988). However, in Charlwood et al. (1988) the mosquitoes in the control and treatment groups were captured and released on different nights. Thus, uncontrolled factors could have influenced the groups of mosquitoes differently on each of the nights. Reanalyzed results showed no statistical differences between the groups' behaviors, nullifying any evidence suggesting that *An. farauti* has a spatial memory (Alonso and Schuck-Paim, 2006). Hii et al. (1991) suggest that *An. balabacensis* prefer to feed on hosts where a previously successful blood meal was had, but the data were not sufficient to determine the extent to which this behavior was innate or learned (Alonso and Schuck-Paim, 2006). On the other hand, McCall et al. (2001) found that *An. arabiensis* showed a significant tendency to return to a house in which they had previously fed, successfully indicating the existence of a retained spatial or olfactory memory.

An. gambiae is one of the main vectors of malaria in equatorial Africa. To date, no known studies have been conducted to determine this animal's learning or chemical

distinguishing capabilities. Except for McCall et al. (2001), previous learning studies conducted with other *Anopheles* species have been inconclusive, but do provide a basis from which to study *An. gambiae*. The ability and extent to which experience influences a vector's choice of resources, such as host and food resources and oviposition and resting sites, can affect its potential for disease transmission (Alonso and Schuck-Paim, 2006). Thus, demonstrating that these medically important mosquitoes can associatively learn odors could lead to a better understanding of their resource locating and choosing abilities (Tomberlin et al., 2006). The improvement this offers to our understanding of the mechanisms underlying heterogeneous patterns of biting activity in mosquitoes amongst their hosts (Alonso and Schuck-Paim, 2006) could lead to new methods of control by manipulating events that determine the vectors' ultimate preferences, thereby reducing the probability that mosquitoes feed on human or other animal hosts (McCall and Kelly, 2002).

To investigate the practicality of the methods of Tomberlin et al (2006) to be adopted for testing associative learning in *An. gambiae*, *Cx. pipiens* mosquitoes, in place of *Cx. quinquefasciatus*, were conditioned to associate a target odor with a sugar-meal using methods adapted from their study. The innate responses of *An. gambiae* to odor compounds were assessed, and the learning abilities of *An. gambiae* to associate a conditioned stimulus (odor) with an unconditioned stimulus (sugar-meal) were examined.

Materials and Methods

***Experiment 1*: Practicality of Tomberlin et al (2006) methods:**

Insect:

The study mosquito *Culex pipiens* was collected at Don Scott field, Columbus, OH in 2008 and reared in the vector behavior laboratory of The Ohio State University (80% ± 5% RH, 27ºC ± 2ºC, and 16:8 (L:D)). Adults were provided with water and a 10% sucrose solution every day, and were given a blood meal once a week by exposing chicken legs to the mosquitoes (OSU animal use protocol number: 2005A0054). Four days after each blood-feeding, a cup for oviposition was placed inside the cage. Two hundred hatched larvae were placed in a pan (23 x 33 x 5 cm) with 450 mL of aged tap water. The larvae were fed powdered Tetramin fish food according to a daily regimen until they developed into pupae. The day they were panned out, they were fed 50 mg, then 100 mg, 300 mg, 300 mg, 500 mg, and then no food for each subsequent day. Following the day that the larvae were not fed, approximately 200 pupae were transferred into a small acrylic plastic cage (20 x 26.5 x 14.5 cm) to emerge, and the adults were provided with a 10% sucrose solution for the first 2 days after emergence and water at all times. Five-day-old, 3-day-unfed *Cx. pipiens* were used for the experiments.

Odor Compounds:

Two compounds were used in this study as both target and non-target odors. Vanilla extract (Rodelle Organic ™, Ft. Collins, CO) was used in this experiment due to its use in previously conducted associative learning experiments with the Braconid wasp *Microplitis croceipes* (Lewis and Takasu, 1990) and *Cx. quinquefasciatus* (Tomberlin et al., 2006). The second compound used was almond extract because it contained the same amount of ethanol as vanilla. Strawberry

extract was not used like in Tomberlin et al. (2006) because it was a proprietary mixture that included vanilla extract.

Conditioning and Testing Procedure:

In general, the mosquitoes were conditioned in an attempt to determine if they could associate an odor with a reward (i.e. a sugar-meal). Association was defined as mosquitoes learning to recognize and respond to a target odor (conditioned stimulus) in association with a sugar-meal (unconditioned stimulus) (Tomberlin et al., 2006). The experiments were conducted between 2:00 pm and 7:00 pm in the vector behavior laboratory (RH: 50% ±5%, temp: 27ºC ± 1ºC) of The Ohio State University. Disposable gloves were worn during all steps of the experiment.

The mosquitoes were individually placed in clear plastic tubes that were 75 mm long and 40 mm in diameter. They were transferred from the acrylic plastic cages to the tubes by an aspirator. Both ends were covered with netting. The mosquitoes were given at least 15 min to acclimate to the environment before the experiment began. Individual mosquitoes were trained by feeding the mosquito from the tip of a 50-µL micropipette with a 10% sugar solution on the inside of the micropipette tip. The target compound coated approximately 1 cm of the micropipette tip. The mosquito was allowed to feed on the sugar solution for 10 s during each of the three feeding intervals, with each interval separated by approximately 30 s (Tomberlin et al., 2006).

Approximately 2 min elapsed between when the mosquito was trained and tested (Tomberlin et al., 2006). To test the trained mosquito, it was presented with a sterile micropipette (blank) as the control, a micropipette coated with ethanol, a micropipette coated with the non-target odor, and a micropipette coated with the target odor in that order (Tomberlin et al., 2006) that were held about 7 mm in front of the proboscis of the mosquito. Each

micropipette was presented to the mosquito for 15 s. An interval of approximately 5-15 s was in between the presentation of the micropipettes. Ethanol was tested because it was the main ingredient of both extracts (approximately 34%). The micropipettes were used once and discarded. The plastic tubes were washed with scalding water and the netting was washed with acetone and scalding water, air dried, and reused. At least 24 hours passed between the washing of the tubes and netting and reusing them.

Behavorial-Response Criteria to Odors:

The responses of the mosquitoes were defined as either positive or negative. A positive behavioral response by the conditioned mosquitoes was defined as the mosquito walking toward the odor source and probing outside or within the tip of the micropipette with its proboscis within the 15 s period of exposure period to the micropipette, and a negative response was defined as the mosquito moving away from the odor source or remaining stationary for more than 15 s while exposed to the micropipette (Tomberlin et al., 2006).

Statistical Analysis:

Cochran's Q test was run to determine if the mosquitoes responded differently to the odors. The confidence interval was 95% for all tests. The McNemar test was used to compare the groups.

Experiment 2: Associative Learning in* Anopheles gambiae*:

Difficulties with the experimental design adapted from the methods presented in Tomberlin et al. (2006) arose. These problems were remedied in the next experiment.

Insect:

The study mosquito *Anopheles gambiae* Mbita strain was obtained from a colony established by the staff of International Center of Insect Physiology and Ecology (ICIPE) in 2001 from a population of *An. gambiae* s.s. in Mbita Point, Kenya. It was obtained and has been maintained in the vector behavior laboratory of The Ohio State University (70% ± 5% RH, 26ºC ± 2ºC, and 12:12 (L:D)) since 2006. Adults were provided with water and a 10% sucrose solution every day, and were given a human blood meal once a week (IRB permit number: 2004H0193). Three days after each blood-feeding, a cup for oviposition was placed inside the cage. One hundred hatched larvae were put in a pan (23 x 33 x 5 cm) with 450 ml of aged tap water. The larvae were fed powdered Tetramin fish food according to a daily regimen described by Gary and Foster (2001). Approximately 100 pupae were placed in a small mouse cage (20 x 26.5 x 14.5 cm) to emerge and the adults were provided with water but not sugar. The *An. gambiae* adults used for the experiments were 12- to 24-hours-old.

Experiment 2-Part A: Determining Odor Compounds for Conditioning:

Individual compounds as opposed to mixtures were needed for conditioning to be able to more effectively and definitively analyze and interpret the results. Honey was chosen to be analyzed because mosquitoes have already shown an innate attraction to it in previous studies (Hancock and Foster, 1997; Foster and Takken, 2004) as well as other experiments in our laboratory (personal communication). A locally obtained honey (Great Value: clover honey [Wal-Mart

Inc., Bentonville, AR]) was analyzed by B. Ebrahimi and P.L. Phelan at The Ohio State University, in connection with another project using the standard techniques for GC-MS. Two g of honey was placed in a 100-ml beaker. This was placed in a ca. 1-L glass container with a teflon lined rubber septum at 30°C for 10 min. Then, a divinylbenzene/carboxen (50/30µm) on polydimethylsiloxane solid-phase microextraction (SPME) fiber (Supelco) was exposed to the honey headspace at 30°C for 10 min by inserting an injector needle through the septum. The fiber was retracted into the SPME injector needle, then inserted into the injector port of a gas chromatograph (GC) for desorption. An Agilent 6890 series GC (Agilent Technologies, Little Falls, DE, USA) coupled with a mass spectrometer (5973 MSD) system equipped with a 7683B series spilt/splitless injector, was used to analyze the volatiles on the SPME fiber. The SPME fiber was desorbed at 225ºC for 3 min in the splitless program mode into a Zebron™ ZB-1 Dimethylpolysiloxane fused column of 30 m length, 0.25mm internal diameter and 0.25µm phase thickness. The oven temperature was held at 25ºC for 2 min, then ramped at 12.5ºC per minute to 240ºC where it was held isothermally for 15 min. The total run time was 20 min and the carrier gas was helium at a flow rate of 1 mL/min. Injector and detector temperatures were maintained at 225ºC.

Innate Responses of Anopheles gambiae *MBITA to Analytical Grade Standard of Honey Headspace Compounds:*

Twelve- to 24-hours-old, unfed *An. gambiae* were individually placed in 75 mm long plastic tubes with netting on both ends. They were transferred from acrylic cages to the tubes with an aspirator. They were then given at least 15 min in the tube for acclimation to the environment before the start of the experiment. The experiments were carried out from approximately 2:00

pm to 6:30 pm. The handling methods and physical conditions of the training and testing environment were the same as for the *Cx. pipiens* experiment.

Each mosquito was presented with a 50-µL micropipette with filled with 1 - 5-µL of ethanol, pentane, benzaldehyde, decanal, nonanal, geranylacetone, phenylacetaldehyde, (Z)-β-ocimene, 3-furaldehyde, or a sterile pipette (blank) for a control. Ethanol and pentane were tested to determine if they could be used later to dilute the main compounds. The micropipettes were placed approximately 7 mm away from the mosquitoes' proboscises. If the mosquitoes were resting on or near the netting, the micropipettes were outside the netting. If the mosquitoes were resting inside the tube further, the micropipettes were careful placed into the tube through holes in the netting in attempts to keep the micropipettes from touching and contaminating the netting. The micropipettes were used once then discarded. The plastic tubes were washed with scalding water and the netting was washed with acetone and scalding water, air dried, and reused. At least 24 hours passed between the washing and reusing of the tubes and netting.

A total of 96 males and 91 females were tested. For each compound, between 10 and 20 total mosquitoes, males and females, were tested. Nine mosquitoes total were tested with the blank.

Behavorial-Response Criteria to the Compounds:

The mosquitoes reactions were monitored for 30 s, and the time between the presentation of the compound and the reaction of mosquito was measured. The responses were defined as positive, negative, or neutral (no response). A positive (or attractant response) was defined as the mosquito probing or moving towards the source of the odor. A negative (or repellent response) was defined as walking or flying away from the presented compound. A neutral response was

defined as remaining stationary for the entire 30 s and was recorded as 60 s for statistical analysis ease.

Statistical Analysis:

A univariate analysis of variance (ANOVA) with a Tukey *post-hoc* test was run to determine statistical differences in mean reaction time of mosquitoes to the different compounds.

Experiment 2-Part B: Determining Associative Learning Capacity in Anopheles gambiae*:*

Odor Compounds:

Of the eight honey compounds, geranylacetone (Aldrich, 65%) was used as the non-target odor and phenylacetaldehyde (Aldrich, 90%) was used as the target odor. They were chosen because, in the previous experiment, the mosquitoes' mean reaction times for these two chemicals were not significantly different from each other and did not indicate either a strong attraction or repulsion response.

Conditioning and Testing Procedure:

Two sets of experiments were conducted in a dual-port olfactometer with static air. It was constructed based on a modified Geier's design (1999). The size of the mixing box in between the release chamber and choice ports was 18 x 10 x 10 cm. One World Health Organization (WHO) test tube was used as a release chamber and two WHO test tubes were used as choice ports in the olfactometer. A funnel trapping system was at the entrance of each of the choice ports. The experiments were carried out from about 5:00 pm to 7:00 am in the dark. The handling methods and physical conditions of the training and testing environment were the same as for the previous experiments.

Five replicates were tested without training to look at possible innate responses that may have been missed in the previous experiment. In each replicate, 7 to 26 12- to 24-hours-old, unfed *An. gambiae*, males and females, were placed in a WHO test tube using an aspirator and given 15 min for acclimation. To prepare the choice ports, ca. 200 ng of phenylacetaldehyde (diluted to 0.1 mol in distilled water) and shaken was impregnated on one 14 x 10 cm filter paper. The filter paper was then placed in one clean WHO test tube. The same method was used to prepare the other port with 200 ng of geranylacetone. After connecting the ports to the olfactometer, the mosquitoes were released and their positions within the olfactometer were recorded 0.5, 1, 1.5, and 14 hours.

Five replicates of 7 to 21 trained mosquitoes, male and female, were carried out. The method just described was followed with one exception: the mosquitoes were trained with phenylacetaldehyde before being released into the olfactometer. To train the mosquitoes, those in the WHO tube for acclimation were transferred to another WHO tube lined with a filter paper impregnated with 200 ng phenylacetaldehyde (target compound). They were exposed to the chemical in this tube for 15 s. The mosquitoes were then transferred to another clean WHO tube lined with a filter paper impregnated with 3-mL of a 10% sucrose solution. The mosquitoes were exposed to the sugar for 30 s. The sucrose filter paper was allowed to dry before training the mosquitoes so they would be able to taste the sugar without being able to ingest it. The mosquitoes were exposed alternately to the phenylacetaldehyde and sugar two more times for a total of three reinforcements. They were transferred from tube to tube with a small fan that sucked them from one tube into the other. After these exposures, the mosquitoes were placed back into the original WHO tube they had been acclimated in before the training. Five minutes after the end of training, the mosquitoes were released in the olfactometer. Filter papers

impregnated with phenylacetaldehyde and geranylacetone were placed one in each of the choice ports. The mosquitoes' position within the olfactometer was recorded at 0.5, 1, 1.5, and 14 hours after release.

Statistical Analysis

A Wilcoxon test for non-parametric paired data was conducted to determine statistical differences in the mean proportion of mosquitoes attracted to the two ports.

Experiment 2-Part C: Determining Odor Compounds for Conditioning:

Innate Responses of Anopheles gambiae *MBITA to Diluted Honey Compounds:*

The innate responses of *An. gambiae* to the honey compounds were reassessed. The compounds used in the previous innate response experiment (Experiment 2-Part A) were the analytical grade standard compounds, which were not the same concentrations as are found in honey. Since it is possible that mosquitoes respond differently to different concentrations of the same chemical, the honey compounds were diluted to the average amount of all the compounds found in honey. The methods were the same as for the first set of innate response experiments (in Experiment 2-Part A). The 50-µL micropipettes that were presented to the mosquitoes were filled with 1 – 5-µL of heptane, mineral oil, an empty sterile micropipette (blank) for a control, or benzaldehyde, decanal, nonanal, geranylacetone, phenylacetaldehyde, (Z)-β-ocimene, or 3-furaldehyde diluted to 0.1 mol in mineral oil. Heptane was tested to determine if it could be used to dilute the main compounds. Ethanol and pentane were not used. For each compound, between 11 to 25 females and 11 to 17 males were tested. Six females and 8 males were tested with the blank.

Behavorial-Response Criteria to the Compounds:

The responses were defined the same as in the previous innate response experiment (Experiment 2-Part A).

Statistical Analysis

The data were not parametric so a Kruskal-Wallis test was conducted to determine statistical differences in mean reaction time of mosquitoes to the different compounds.

Experiment 2-Part D: Determining Associative Learning Capacity in Anopheles gambiae*:*

Odor Compounds:

Of the eight honey compounds, (Z)-β-ocimene (Aldrich, 70%) was used as the non-target odor and linalool oxide (Wako, 98%) was used as the target odor. They were chosen because, in the previous experiment, the mosquitoes' mean reaction times for these two chemicals were not significantly different from each other and did not indicate either a strong attraction or repulsion response.

Conditioning and Testing Procedure:

The modified methods of Tomberlin et al. (2006) were revisited with *An. gambiae*. Two changes were made from Experiment 1 with *Cx. pipiens*. First, the *An. gambiae* mosquitoes were anywhere from 12- to 48-hours old. Second, to test a trained mosquito, it was presented with a sterile micropipette (blank), a micropipette coated with mineral oil, a micropipette coated with the non-target odor, and then a micropipette coated with the target odor in that order. Since linalool oxide and (Z)-β-ocimene were diluted in mineral oil, the mosquitoes were presented with mineral oil for the same reasons ethanol was tested in Experiment 1.

Behavorial-Response Criteria to Target and Non-target Odors:

The responses of the mosquitoes were defined exactly the same as in Experiment 1 with *Cx. pipiens*.

Statistical Analysis

No statistical analyses were done with this set of experiments because data was not obtained due to an inability to train and test the mosquitoes.

Results

***Experiment 1*: Practicality of Tomberlin et al (2006) methods:**

Differences in responses to the odors were seen for females, males, almond extract as target, and vanilla extract as target based on Cochran's Q test (Figure 1). When trained to almond extract, 12.5% of females responded to the blank, 59.38% responded to ethanol, 56.25% responded to the non-target vanilla, and 84.38% responded to the target almond (N=32, Cochran's Q=41.10, df=3, $P<0.0001$) (Figure 1A). Three significantly different groups were found. The responses to ethanol and the non-target were not significantly different ($P>0.05$). When trained to almond extract, 18.18% of males responded to the blank, 63.64% responded to ethanol, 63.64% responded to the non-target vanilla, and 96.97% responded to the target almond (N=33, Cochran's Q=45.60, df=3, $P<0.0001$) (Figure 1B). Three significantly different groups were found. Like the females, the males' responses to ethanol and the non-target were not significantly different ($P>0.05$). When trained to vanilla extract, 4.0% of females responded to the blank, 64.0% responded to ethanol, 88% responded to the non-target almond, and 88% responded to the target vanilla (N=25, Cochran's Q=49.82, df=3, $P<0.0001$) (Figure 1C). Three

significantly different groups were found. Their responses to the non-target and target were not significantly different (P>0.05). When males were trained to vanilla extract, 8.82% responded to the blank, 61.76% responded to ethanol, 76.47% responded to the non-target almond, and 94.12% responded to the target vanilla (N=34, Cochran's Q=59.87, df=3, P<0.0001) (Figure 1D). Three significantly different groups were found. Neither the responses to ethanol and the non-target (P=0.063) nor the responses to the non-target and target (P=0.070) were significantly different at a 95% confidence interval but they were at a 90% confidence interval.

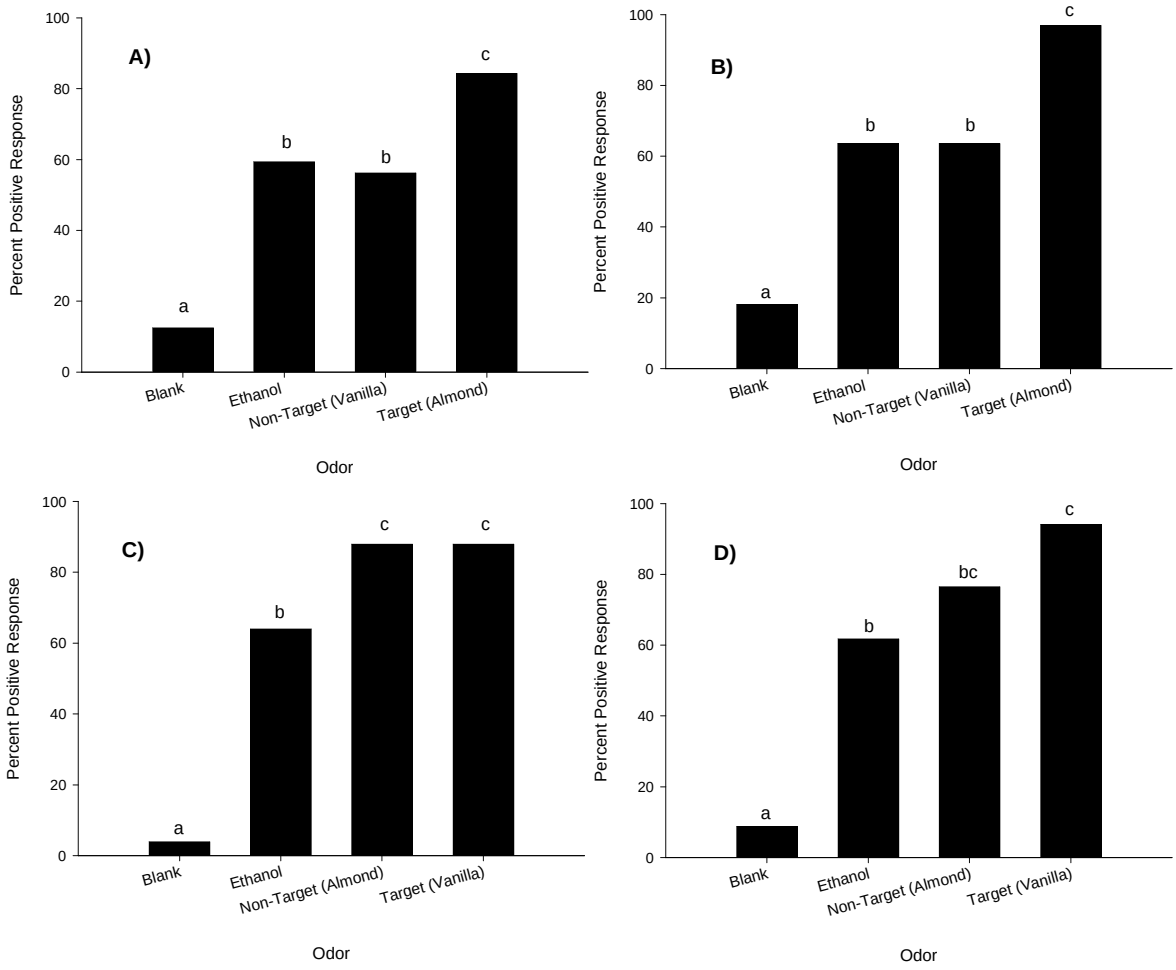

Figure 1: Percent of positive responses of mosquitoes to the four treatments. A) Females with almond extract as target, B) males with almond extract as target, C) females with vanilla extract as target, and D) males with vanilla extract as target. The lettered columns represent the significantly different (P<0.05) groups.

Experiment 2*: *Associative Learning in* Anopheles gambiae*:

Experiment 2-Part A: Determining Odor Compounds for Conditioning:

Using the GC-MS analysis, the chemicals found to be in abundance in honey were 3-furaldehyde (a.k.a. 3-furfural), (Z)- β-ocimene, benzaldehyde, nonanal, phenylacetaldehyde, decanal, geranylacetone, and butylated hydroxytoluene (BHT) (Figure 2). BHT is a common commercial antioxidant that is used as a preservative and was not tested in the following experiments. The GC-MS retention time of these compounds ranged from 2.218 min to 10.679 min, and the relative abundances in honey ranged from 1.7% to 25.6% excluding BHT (Table 1).

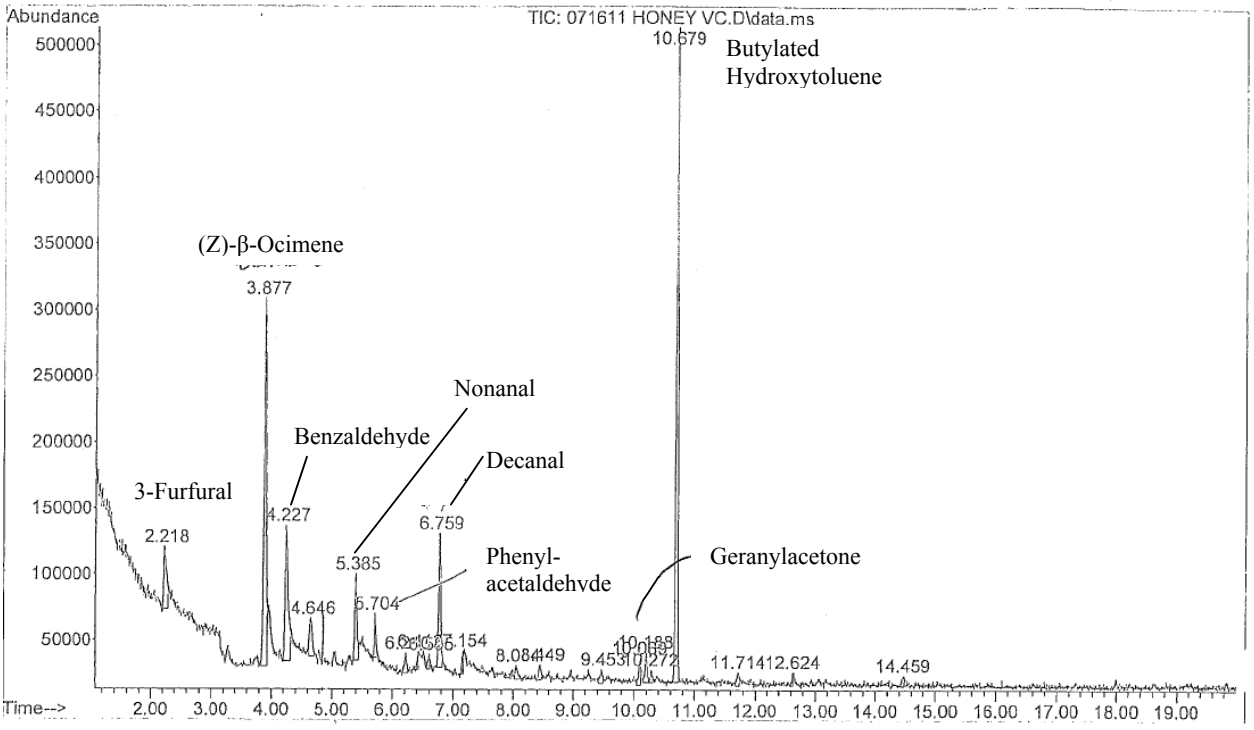

Figure 2: GC-MS for honey with the retention time on the x-axis (min) and the relative abundance on the y-axis.

Chemical	Retention time (min)	Relative Abundance (%)	Molecular Weight
3-Furaldehyde	2.218	5.220	96.08
(Z)-β-Ocimene	3.877	25.648	136.23
Benzaldehyde	4.227	11.240	106.12
Nonanal	5.385	5.876	142.24
Phenylacetaldehyde	5.704	2.688	120.15
Decanal	5.759	7.885	156.27
Geranylacetone	10.188	1.704	194.31
BHT	10.679	34.961	220.35

Table 1: Retention times (min), relative abundances (% area under the curve), and molecular weights for the chemicals found in abundance in honey.

Innate Responses of Anopheles gambiae *MBITA to Analytical Grade Standard of Honey Headspace Compounds:*

The mean response times to analytical grade standards of each compound found in honey headspace, ethanol, pentane, and the blank ranged from 16.1 s to 55.9 s. The smaller numbers indicate shorter response times and faster responses. The mosquitoes' responses were only monitored for 30 s, but a neutral response was recorded as 60 s for statistical analysis ease.

According to the ANOVA, there was no difference between males and females in innate responses to the compounds ($F=0.174$, $df=1$, $P=0.677$). Differences in the mean response time of the mosquitoes to the compounds did occur ($F=3.291$, $df=10$, $P=0.001$) (Figure 3). Three significantly different groups were found. The responses to benzaldehyde, 3-furaldehyde, (Z)-β-ocimene, geranylacetone, phenylacetaldehyde, ethanol, pentane, nonanal, and linalool oxide were statistically similar. The mean responses times for this group ranged from 16.11 s to 38.42

s. The second group was the first group without benzaldehyde and with decanal and had a mean response time range from 24.31 s to 47.68 s. The responses to phenylacetaldehyde, ethanol, pentane, nonanal, linalool oxide, decanal, and the blank were statistically similar as well. The mean response times for this group ranged from 31.11 s to 55.89 s. There was no interaction effect between mosquito sex and compound tested (F=0.646, df=10, *P*=0.773).

From these results, phenylacetaldehyde and geranylacetone were chosen to be used to train and test the mosquitoes in the next experiment. They were chosen because the mosquitoes' mean reaction times for these two chemicals (approximately 28 s) were not significantly different from each other. 28 s is a short enough amount of time to ensure that that they could smell these compounds, but not long enough to have a strong innate attraction or repulsion response.

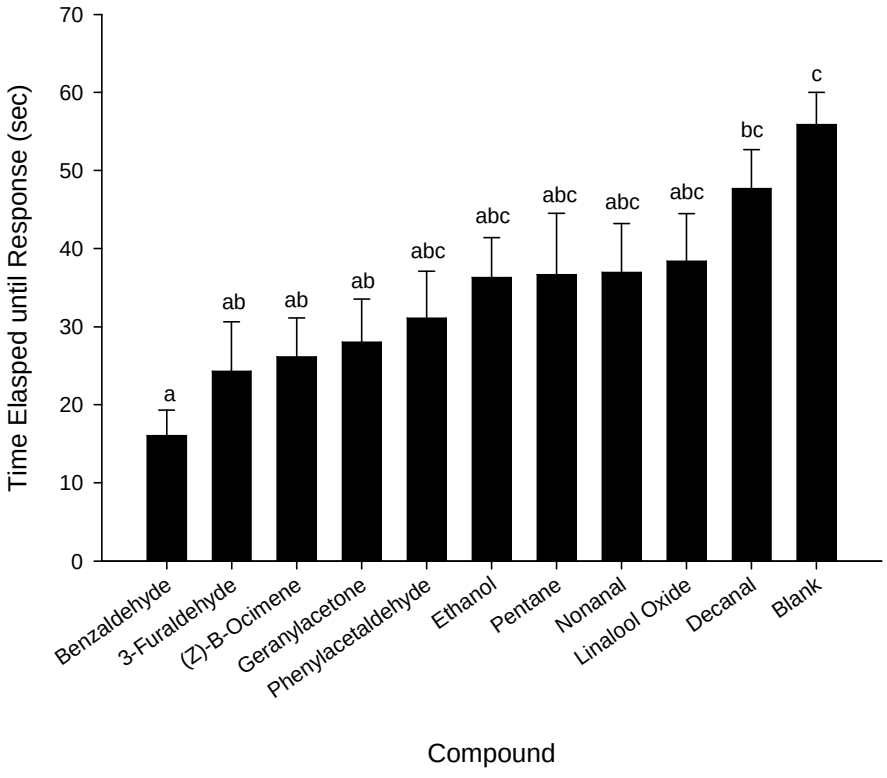

Figure 3: Mean innate response times with standard errors of female and male mosquitoes to the main volatiles of honey, ethanol, pentane, and blank.

Experiment 2-Part B: Determining Associative Learning Capacity in Anopheles gambiae*:*

No response was seen within 1.5 hrs after release of mosquitoes. Therefore, the experiments were run overnight (14 hours) and the mosquitoes' positions within the olfactometer were recorded. 2.99% of the untrained, naïve mosquitoes responded to phenylacetaldehyde and 22.17% responded to geranylacetone (Figure 4A). 2.00% of the trained mosquitoes responded to the target phenylacetaldehyde and 10.46% responded to the non-target geranylacetone (Figure 4B). A discrimination between phenylacetaldehyde and geranylacetone of 0.083 (number of mosquitoes responded to phenylacetaldehyde/number of mosquitoes responded to both phenylacetaldehyde and geranylacetone) was observed in naïve mosquitoes, and a discrimination of 0.167 was observed in mosquitoes trained with phenylacetaldehyde. However, the non-parametric paired Wilcoxon test did not show a statistical difference in the mean proportions of mosquitoes attracted to the two ports with or without training.

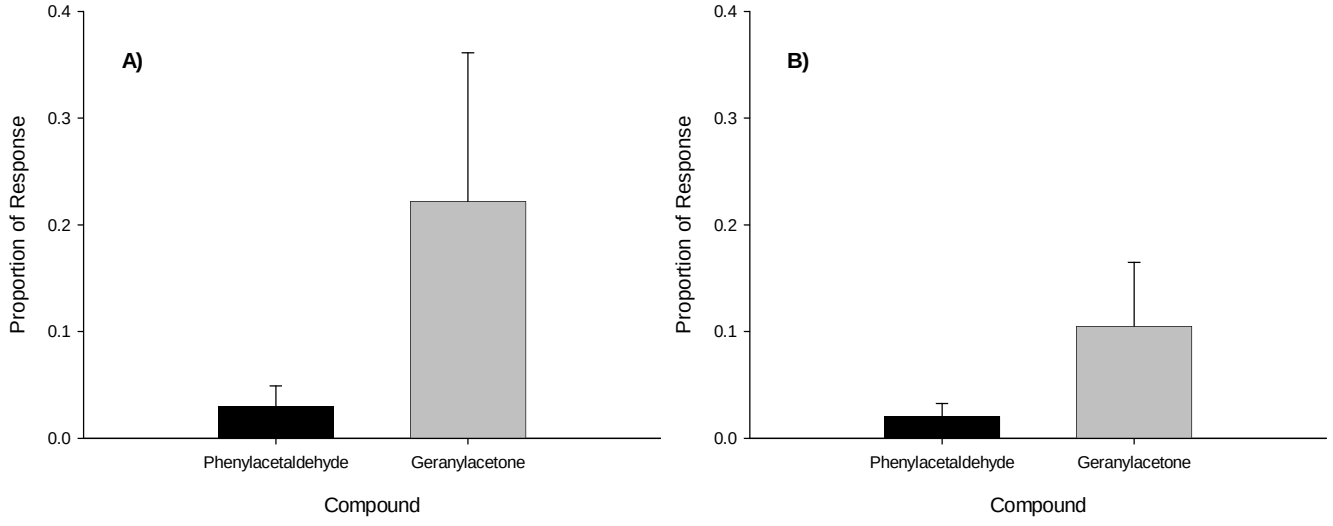

Figure 4: Proportions of responses and standard errors to phenylacetaldehyde and geranylacetone for each replicate after 14 hours in the olfactometer A) without training and B) with training.

Experiment 2-Part C: Determining Odor Compounds for Conditioning:

Innate Responses of Anopheles gambiae *MBITA to Diluted Honey Compounds:*

The mean response times to the diluted honey compounds, heptane, and the blank range from 15.4 s to 43.7 s in females and from 30.1 s to 51.5 s for males. Again, the smaller numbers indicate shorter response times and faster responses. The mosquitoes' responses were still monitored for only 30 s, and a neutral response was recorded as 60 s for statistical analysis ease.

The females and males showed different patterns of responses when analyzed separately. According to the Kruskal-Wallis test, differences in the mean innate response times to honey compounds, heptane, mineral oil, and blank existed for females (Kruskal-Wallis=23.410, df=10, *P*=0.009) (Figure 5A) but not for males (Kruskal-Wallis=11.55, df=10, *P*=0.317) (Figure 5B). Females responded differently to the compounds while the males' responses to all the compounds were statistically the same. Females responded similarly to benzaldehyde, nonanal, phenylacetaldehyde, linalool oxide, (Z)-β-ocimene, heptane, geranylacetone, decanal, and 3-furaldehyde. The other group of compounds with significantly similar responses was the first group without benzaldehyde and with mineral oil and the blank.

From these results, linalool oxide and (Z)-β-ocimene were chosen to be used to train and test the mosquitoes in the next experiment. They were chosen because, in the previous experiment, the mosquitoes' mean reaction times for these two chemicals (approximately 24 s for females and 35 s for males) were not significantly different from each other. Those response times are a short enough amount of time to ensure that that they could smell these compounds, but not long enough to have a strong innate attraction or repulsion response.

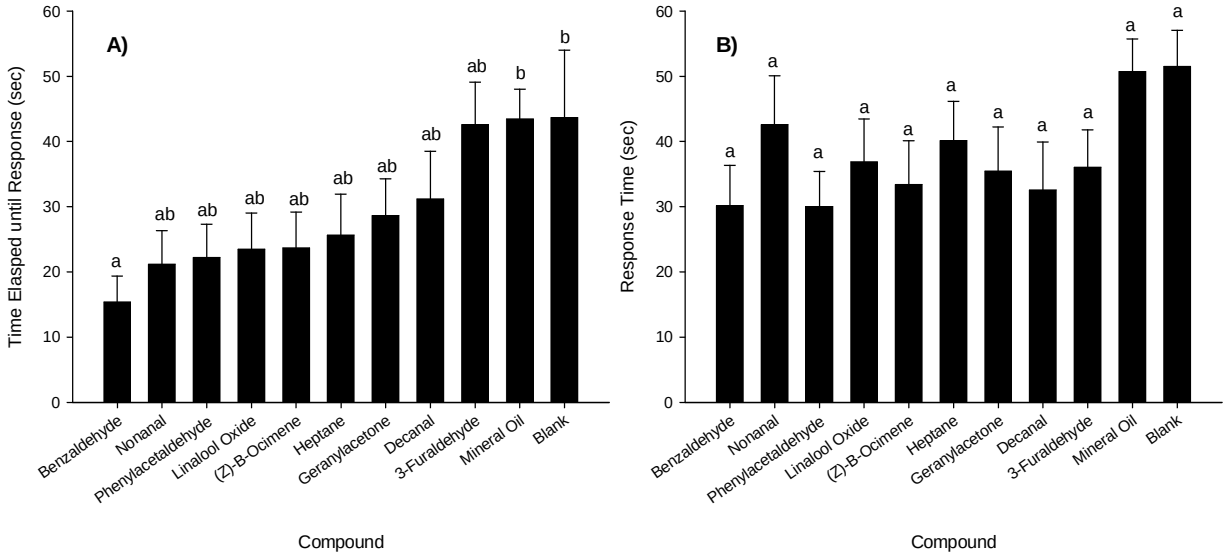

Figure 5: Mean innate response times with standard errors to the main volatiles found in honey diluted to 0.1 mol in mineral oil, heptane, mineral oil alone, and blank for A) females and B) males.

Experiment 2-Part D: Determining Associative Learning Capacity in Anopheles gambiae*:*

Data was not obtained for these experiments because the mosquitoes would not feed on the sucrose solution from the micropipette in the way that *Cx. pipiens* did in Experiment 1. They were extremely skittish and difficult to feed from the pipette. Even when it was possible to gently put the pipette with only the sucrose solution around their proboscises, they were not interested in feeding regardless of their energy levels. This inability to train the mosquitoes left us with no numerical data at the time.

Discussion

Learning is defined as a modification in behavior due to experiences (McCall and Kelly, 2002). Little conclusive evidence exists to demonstrate learning in the medically important mosquito due to experimental design flaws in previous studies brought to light by Alonso and Schuck-Paim (2006). This study provides some evidence for the possibility that the malarial vector *An. gambiae* has the capacity to associatively learn, but, like previous experiments, is not entirely conclusive due to the nature of the experimental design.

When determining the practicability of the methods from Tomberlin et al (2006), several issues arose. The compounds they used as the non-target and target odors were vanilla and strawberry extracts. The strawberry extract used was a proprietary mixture of compounds that includes vanilla extract (Tomberlin et al, 2006). Thus, the mosquitoes could have been responding to both the non-target and the target not because of lack of learning abilities but because the odors were not different enough for the mosquitoes to be able to discriminate between them, they were responding to the components common to both extracts, or a combination of both. In an attempt to remedy this, we used vanilla and almond extracts. However, both of these extracts contained approximately 34% ethanol. We exposed the trained mosquitoes to ethanol as well as the non-target and target compounds to determine if there was any background attraction to ethanol that might skew the responses to the non-target and target compounds; Tomberlin et al (2006) did not do this. Both female and male mosquitoes responded more to ethanol than to the blank (Figure 1). Lefevre et al. (2010) found that beer consumption (containing ethanol) increased human attractiveness to mosquitoes. Ergo, mosquitoes may have an innate attraction to ethanol. This could explain why there is differentiation in the females' responses to ethanol and the non-target when almond extract was the target odor (Figure 1A-B)

and differentiation in the males' responses between the two when the target was vanilla extract (Figure 1D). Another problem with using extracts is that they are not pure; they contain several compounds. It is probable that there were component other than ethanol that were common to both almond and vanilla extracts explaining why the mosquitoes could not distinguish between the non-target and target odors when vanilla was the target (Figure 1C-D). This result is interesting because they could distinguish between the non-target and target when almond was the target odor (Figure 1A-B). This could indicate that mosquitoes are innately attracted to almond extract, or that there is some chemical in common between almond and vanilla extracts that the mosquitoes associate with a sugar-meal when trained with vanilla but not when trained with almond because of other uncommon components. It is quite possible that these mosquitoes, like honey bees, are able to generalize a conditioned response from a mixture to specific compounds found in the mixture (Smith et al., 2006). Thus, the mosquitoes may have been responding to the ethanol or other compounds found in both extracts due either to previous exposure during training or to innate attraction to ethanol as opposed to those chemicals unique to each extract. This could quite possibly skew the results such as we found in following experiments.

Similarly, at low detectable intensities, animals behave as though they can detect the presence of an odor, but have difficulty in determining its identity (Smith et al., 2006). The amount of chemicals applied to the pipette to be presented to the mosquitoes may have been too low for the mosquitoes to be able to detect or differentiate among them. Also, each mosquito was tested with the blank, ethanol, non-target, and target, respectively. Being exposed to the four odors in sequence could have affected the mosquitoes' responses. The blank could have changed the way they responded to ethanol, non-target, and target; ethanol could have changed

their responses to the non-target and target; and being exposed to the non-target could have affected the mosquitoes' responses to the target. Ideally, each mosquito would have been exposed to all four of these odors simultaneously and equidistant from its proboscis. However, this would have been difficult to do adequately with the set-up of the experiment.

We used the Cochran Q statistical test to take into account the nature of the repeated measures in the experiment, where as the PROC GLM test used by Tomberlin et al. (2006) is only appropriate when the data are independent. Also of note is that the responses for males trained with vanilla extract were not significant at a 95% confidence interval but were at a 90% confidence interval. Had more replications been conducted, the results may have shown significant differences. However, it was overly time consuming to obtain ample replicates when training and testing mosquitoes individually. Although, it is important to note that the differences seen between Tomberlin et al. (2006) and this experiment could have been due more to the difference in mosquito species and less to the experimental methods.

Despite these experimental design setbacks, our results indicate that *Cx. pipiens* may be able to associate an odor with food because the responses when almond was the target was significantly greater than for the blank, ethanol, and non-target (Figure 1A-B). However, due to the higher response rate of mosquitoes to ethanol and the non-target when compared to the blank and their inability to distinguish between the target and non-target when vanilla was the target, it cannot be determined what exactly the mosquitoes were responding to.

As previously stated, single chemical compounds, as opposed to mixtures, are needed for these experiments in order to determine exactly what the mosquitoes are responding to. This would ensure that they are not responding to something that is common to both the non-target and target, thus skewing the results. We decided to analyze honey and use its compounds for the

non-target and target odors. The most abundant compounds were 3-furaldehyde, (Z)-β-ocimene, benzaldehyde, nonanal, phenylacetaldehyde, decanal, and geranylacetone (Figure 2). (Recall that butylated hydroxytoluene is also abundant but is a preservative to increase shelf life and was not used in our experiments.) Honey was chosen because mosquitoes have shown an attraction towards it in previous studies (Hancock and Foster, 1997; Foster and Takken, 2004) as well as other experiments in our laboratory (personal communication). However, we want to avoid using chemicals that the mosquitoes have an innate attraction for, like was quite possibility the case with ethanol in the previous experiment with *Cx. pipiens*, because this decreases the researchers' abilities to adequately assess learning capabilities.

We conducted an experiment in order to determine innate responses to the honey components and which ones to use for subsequent associate learning experiments. Phenylacetaldehyde, a compound that has successfully been used in mosquito learning (Jhumur et al. 2006), and geranylacetone, a compound with a very close innate response to phenylacetaldehyde, were chosen because they both have an intermediate response time of around 30 seconds (Figure 3), indicating, as previously stated, that the mosquitoes can smell them but have no strong attraction or repulsion response. Also of note is that there was a somewhat innate response to ethanol (Figure 3), as was observed in Experiment 1 with *Cx. pipiens*, further supporting our hypothesis that the ethanol in the almond and vanilla extract in the previous experiments is partially responsible for the observed results.

Naïve mosquitoes were tested in the olfactometer to determine if there was any innate response to phenylacetaldehyde and geranylacetone missed by the previous experiment and for comparison with the trained mosquitoes. They seemed to be slightly innately attracted to geranylacetone (Figure 4A) even though no significant difference exists between the proportion

of mosquitoes attracted to phenylacetaldehyde and geranylacetone. Thus, if there is an innate attraction or repellant to these compounds, it is relatively equal, meaning that these compounds are good to use for comparison. Like the naïve mosquitoes, the trained mosquitoes did not show any discrimination between phenylacetaldehyde and geranylacetone (Figure 4B). However, the discrimination between the two compounds for trained mosquitoes was twice that of naïve mosquitoes (0.167 vs. 0.083) even though the statistical test for proportion of caught mosquitoes in each port did not show any statistical difference. Thus, it is plausible that slight amounts of learning may have occurred. Also of note is that the proportion of mosquitoes attracted to geranylacetone was greater in naïve mosquitoes than in trained mosquitoes. Even though there is no statistical difference, it shows that the training process may have had some effect on the mosquitoes' responses to phenylacetaldehyde and geranylacetone.

In the olfactometer experiment (Experiment 2-Part B), the compounds were diluted in water to 0.1 mol, the average amount of the compounds found in honey, whereas in the innate response experiment (Experiment 2-Part A) to determine which compounds to use for the olfactometer experiment, the pure compounds were used because we were still attempting to determine what to dilute the compounds in. This could have caused the innate responses of the olfactometer experiment to be different from the first innate response experiment. Training the mosquitoes en masse, although allowing for more replications in less time, was very disturbing to the mosquitoes. They were constantly being forced from one WHO tube to another. The disturbance of training could have hindered the mosquitoes' abilities to learn. Plus, olfactometer experiments typically only produce an average of response of 30% (Geier et al., 1999; unpublished data from our laboratory). Low responses rates hinder the ability of statistical analyses to recognize differences that may actually exist. Also, mosquitoes need to be at an

optimal physiological hunger state for these experiments (Jhumur et al., 2006). If the mosquitoes are not hungry enough, they will not want the sugar-meal so may not be able to be trained and will not respond when tested. If the mosquitoes are too famished, they will not have enough energy to respond even if they did learn to associate the odor with a sugar-meal. These inadequate hunger states may not only affect the responses of the mosquitoes, but may also affect their learning abilities.

The olfactometer experiment has not shown that *An. gambiae* can learn. However, the doubled discrimination of trained mosquitoes in comparison to naïve mosquitoes indicates that with more experiments, more and/or different training trials, shorter time between conditioned and unconditioned stimuli, more replicates, bigger sample size, mosquitoes in the optimal physiological state, or standardized concentration of compounds, the learning abilities of *An. gambiae* could be determined.

We conducted innate response experiments again with the compounds diluted to 0.1 mol—the average amount of the compounds present in honey—in mineral oil because innate responses to chemicals can differ as concentration changes. For example, low concentrations can be undetectable and high concentrations can be repellent in some mosquitoes (Logan et al., 2008). Mineral oil was used as the dilutant because it is nonpolar like the honey compounds and has no noticeable odor to humans. Based on the results of this experiment, linalool oxide and (Z)-β-ocimene were chosen to be used for the next set of associative learning experiments because of their intermediate mean innate response times (Figure 5). Even though the responses of female and males differed, the responses to linalool oxide and (Z)-β-ocimene were similar enough to be comparable and used for females and males alike.

Due to the lack of conclusive results in the previous olfactometer experiments, as previously described, we decided to go back to the revised Tomberlin et al (2006) methods with *An. gambiae* using linalool oxide and (Z)-β-ocimene, single chemical compounds, instead of extracts, which is where most of the difficulties arose previously. However, different setbacks arose with *An. gambiae*. They were extremely difficult to train, much more so that *Cx. pipiens*. This could have been due to their naturally skittish behavior or their lack of hunger. In an attempt to find the optimal hunger state, we attempted to train and test mosquitoes that were approximately 1, 2, and 3 days old. The best and most conclusive results were observed with approximately 3-day-old mosquitoes, but most starved *An. gambiae* die before reaching that age. It was not possible to obtain enough replicates under the time constraints of the experimenter to obtain results or come to any conclusions about this set of experiments. Even when *An. gambiae* mosquitoes were able to be trained, the testing procedure was inconclusive. An adequate amount of reinforcements of the stimuli are needed in order to instill association (Smith et al., 2006). Three 10 s exposures may not have been enough reinforcements for *An. gambiae* to associatively learn an odor with a sugar-meal. Likewise, two 70-min exposures conducted in an experiment by Alonso et al. (2003) may not have been adequate to elicit an associative learning response in *Ae. aegypti*. Other experiments with *Anopheles* spp. have had similar difficulties with obtaining conclusive data. According to Alonso and Schuck-Paim (2006), the data produced by Hii et al. (1991) suggesting that *An. balabacensis* prefers to feed on hosts where a previously successful blood meal was had were not sufficient to determine the extent to which the observed behaviors were innate or learned.

In summary, future studies should optimize the physiological hunger state to enhance the mosquitoes' responses. Also, an effective method of en masse training and testing should be

used in order to obtain many replicates. However, individual mosquito training and testing enables the researcher to know whether or not each mosquito has been adequately training and is able to be tested whereas with training en masse, knowing whether or not each mosquito has been adequately trained is difficult to determine. Furthermore, suitable compounds need to be employed. Although our methods used to determine appropriate compounds to use were adequate, ideally we would have been able to know for certain if the mosquitoes could smell the compounds presented to them or not. This could be done with a joint gas chromatograph – electro-antennographic (GC-EAG) technique. Additionally, the stimuli need to be reinforced an ample number of times. As stated previously, inadequate reinforcement may have caused the inability of Alonso et al. (2003) to determine learning abilities in *Ae. aegypti*. Lack of sufficient reinforcement in both the experiment with *Cx. pipiens* and the olfactometer experiment with *An. gambiae* could have contributed to our own inconclusive results.

Based on the results obtained, there is evidence to believe that *An. gambiae* mosquitoes can learn to associate an odor with a sugar-meal. We cannot state that they cannot associatively learn because of the flaws in the experimental designs and due to the fact that claims for lack of learning in animals is problematic (Dukas, 2008). This is because the animals might have low motivation or behavioral deficiency caused by the experimental settings rather than a genuine inability to learn (Dukas, 2008). Several previous studies looking at mosquito learning, as reviewed in the introduction, have had difficulties with experimental design making it difficult to come to a firm conclusion about learning capabilities (Giglioli, 1964; Snow, 1970; Charlwood et al, 1988; Hii et al, 1991; Kaur et al, 2003). Also, since fruit flies likely do not possess exceptional learning abilities relative to other insect taxa, it is safe to assume that most other insects also commonly employ learning in all central aspects of life (Dukas, 2008). Due to the

inherent nature of these types of experiments, it is necessary in future studies to ensure that all the parameters that may affect the ability of mosquitoes to learn are taken into account in the experimental design so that definitive conclusions can be made. The importance of conclusively determining if *An. gambiae* mosquitoes can learn is paramount when creating better methods for control and fully understanding the realm of their vectorial capacity.

Acknowledgements

I would like to thank Dr. Woodbridge Foster and Babak Ebrahimi for their continued guidance and assistance throughout the project, and Dr. P. Larry Phelan for his correspondence with regards to chemical compounds and experimental design. I would also like to thank every member of the Vector Behavior Laboratory at The Ohio State University for their encouragement and support along the way.

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